Effect of immobilization on production of ethanol using yeast cells

For partial fulfillment for the award of the Degree of

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IN

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Under the esteemed guidance of

Dr. R. Jayabalan

Submitted by

Jijnasa Barik

(Roll no.-412LS2033)

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA – 769008

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राष्ट्रीय प्रौद्योगिकी संस्थान
NATIONAL INSTITUTE OF TECHNOLOGY
राउरकेला ROURKELA - 769008, ओडिशा ODISHA

RIGHT TO INFORMATION

Dr. R. Jayabalan
Assistant Professor
Department of Life Science

Date: 11/05/2014

CERTIFICATE

This is to certify that the thesis entitled "**EFFECT OF IMMOBILIZATION ON PRODUCTION OF ETHANOL USING YEAST CELLS"** which is being submitted by **Ms. Jijnasa Barik**, Roll No. **412LS2033**, for the degree of Masters of Science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

R. Jayabalan 11/05/2014

R. Jayabalan.

DECLARATION

I hereby declare that the thesis entitled "**Effect of immobilization on production of ethanol using yeast cells**" submitted to the Department of LIFE SCIENCE, National Institute of Technology, Rourkela for the partial fulfilment of the Master of Science in Life Science is a faithful record of original research work carried out by me under the guidance and supervision of Dr. R. Jayabalan, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: 11/05/2014

Place: Rourkela

Jijnasa Barik

Jijnasa Barik

(Roll No. 412LS2033)

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

g	**Gram**
mg	**Milligram**
L	**Litre**
ml	**Millilitre**
º	**Degree**
C	**Centigrade**
h	**Hour**
Min	**Minute**
Psi	**Pressure per sq. inch**
%	**Percentage**
MGYP	**Maltose Glucose Yeast Extract Peptone**
M	**Molar**
mM	**Millimolar**
rpm	**Rotations per minute**
CFU	**Colony forming units**
RT	**Room temperature**
FE-SEM	**Field Emission Scanning Electron Microscopy**

List of Contents

Abstract

Sugar is converted to ethanol and CO2 by yeast during the fermentation process. Ethanol has various uses like it used as fuel, help in preserving biological sample, used as solvent, manufacture of acetaldehyde, ethanoic acid, etc. Use of yeast cells in fermentation industry on economic grounds proposes entrapment of yeast cell and their utilization. Immobilization of yeast cells offers many advantages as compare to free cells like easy recovery of product, its cost effectiveness, stability, yeast cells can reuse in further fermentation process etc. In this study, three yeast strain *Saccharomyces cerevisiae* NCIM 3570, *Candida shehatae* NCIM 3500, *Dekkera naardenensis* NCIM 3575 were immobilized using 3% Sodium alginate. The calcium alginate beads thus formed were used as inoculums for ethanol production independently where free cell inoculums of respective strain were taken as control. The initial glucose concentration in fermentation media for *S. cerevisiae* and *D. naardenensis* was 10% whereas for C. shehatae it was 5% glucose along with 5% xylose. Amount of ethanol was estimated by HPLC analysis using Hiplex-H Agilent column having Column and Detector Temperature 57°C and 50°Crespectively, flow rate 0.7 ml/min, mobile phase 1 mM H2SO4 using refractive index detector. Also, viability of immobilized yeast cell stored in different condition like 4°C, room temperature, used bead at 4°C for 45 days was checked by plating on MGYP agar plates. Effect of immobilization on ethanol production was compared between both free cell and encapsulated cell. Ethanol concentration in immobilized *S.cerevisiae* was found to be higher (194.69) g/l as compared to the free cell (81.24 g/l). In case of *D. naardenensis* and *C. shehatae* the ethanol produced in immobilized cell is less as compare to free cell. Good numbers of colonies were found in case of 4°C, room temperature and one time-utilized beads stored at 4°C was checked after 45 days and found that the cells retained viability**.**

Key words- Yeast cells; immobilization; fermentation; encapsulated cell; HPLC

1. Introduction

Immobilization is the imprisonment of all types of biocatalysts including enzymes, cellular organelles, animal and plant cells in a distinct phase that allows exchange with but is separated from the bulk phase or the external environment. Immobilization is the technique used for the physical or chemical fixation of cells, organelles, enzymes, proteins onto or into a solid support, or retained by a membrane, so that their stability is increased and it make possible their continuous use. Immobilization has a wide range of application in many industries like biotechnology, pharmaceutical, environmental, food and biosensor industries. This technique possesses many benefits over free cells.

1.1 Types of immobilization

Immobilization techniques can be widely done by using four main methods based on the physical mechanism employed: first, is attachment or adsorption on solid carrier surfaces, which, is the simplest method of reversible immobilization commonly, used for attachment of cell. This method is easy in preparation and based on weak forces. Second, is entrapment within a porous matrix that is an irreversible method of immobilization where the membrane itself is semi permeable that allows substrates and nutrients in and out of the membrane. Third, is self-aggregation by flocculation, which is natural, or with cross-linking agents, which is artificially induced. This technique is achieved by increasing the effective size or density of cell by aggregation. It can be natural or artificially induced by cross-linking agents and the fourth one is cell containment behind barriers (Lee et al., 2011)

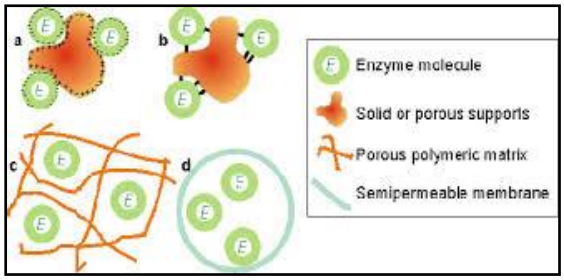

Fig. 1: Types of immobilization (a) adsorption over solid surface, (b) covalently bound to the carrier surface, (c) entrapment within porous matrix and (d) cell enclosed in barriers

Among these, encapsulation is the most extensively studied method due to its simplicity and operationally convenient workup. Encapsulation refers to a physicochemical or mechanical process to entrap a substance in a material thus the produced particles are with diameters of a few nanometers to a few millimeters. It can also be described as the entrapment of microbial cell in polymer matrices. So here, the active agents are entrapped within the polymer matrix or the capsules. In such case cells are restricted by the membrane walls that is in a capsule, but free-floating within the core space. This technique creates a protective barrier around the entrapped microbes which ensures their prolonged viability during processing and storage in polymers (Martins and Santaella, 2013)

1.2 Encapsulating matrices

Encapsulation matrices include agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. Polyacrylamide, alginate, and carrageenan are the commonly used polymer matrices. Chitosan is a naturally occurring polymer, derived from chitin, shell waste of crustaceans. Chitin is composed of 5000- 8000 residues of highly ordered β-(1,4)-N-acetyl-D-glucosamine (2-acetamido-2-deoxy-δ-glucose).It used in both entrapment and encapsulation coating. A thermally and mechanically stable beads is produced in which amine groups of chitosan bind strongly to alginate carboxylic groups .Carrageenan is an copolymer of 1, 3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-D-galactose.In this case gel formation is temperature dependent and reversible. Aggregation of chain in desired confirmation can be promoted by potassium ion as they harden on cooling. Cellulose is the most common natural polymer composed of 1,4-linked β-D-glucopyranosyl chains which is additionally bind with hydrogen bonds. Maltodextrin is also a polysaccharide derived from starch is a suitable coating material in microencapsulation by spray drying. Synthetic polymer like polyacrylamide, polyurethane is also used for cell immobilization. Granules formed by poly acrylamide gel are more resistant and they keep their shape during the fermentation process.

1.3 Properties of encapsulating matrices

The matrices used in encapsulation technique should be semi permeable, thin but strong to support the environmental conditions, should not react with the substrates, nutrients or products, have functional group for cross linking, permeable to reactant and product, elastic enough to accommodate growing cells, should not reduce the biocatalytic activity of cell, resistant to microbial degradation, retain their chemical and thermal stability during the bioprocess . The use of free cell in industrial application has much limitation for example their instability and irrecoverability (Meena and Raja, 2004). Encapsulation in beads offers numerous advantages in contrast to free cells. It reduces the cost of bioprocessing due to cell reuse in subsequent reaction cycles, and a reduced possibility of contamination, provides a minimal cost of separation, provides resistance to shear for shear sensitive cells, maintain a high cell density, it eliminates the long and expensive process of separating enzyme substrates separation and their purification.This method is preferable for cell immobilization because its preparation exhibits decreased cell leakage and has high loading capacity (Cardona and Sanchez, 2007). This method is easy because a wide variety of natural and synthetic polymer can used in this process.

1.4 Alginate

The most suitable biomaterials for encapsulation technique due to their abundance, excellent biocompatibility and biodegradability properties is the alginate. Entrapment within the natural polymer i.e. calcium alginate is the most widely used technique for immobilising cells. Large scale of calcium alginate bead can produce without any sophisticated instrument and these gels are also thermostable at a temperature range of 0-100°C. Alginates are commercially available as water-soluble sodium alginates and it is a rapid, nontoxic, inexpensive and versatile method for immobilization for cells (Fraser and Bickerstaff, 1997).It is also reversible, and has good mechanical properties (Margaritis and Kilonzo, 2005) .Calcium alginate bead is one of the most commonly used support for the immobilization of cells and it not only easy to carry out but also provides extremely mild conditions, so that it have a higher potential for industrial application . Alginate is a naturally occurring marine polysaccharide extracted from various species of brown algae (Phaeophyceae), including *Ascophyllum*, *Laminaria*, *Lessonia*, *Ecklonia*, *Durvillaea* and *Macrocystis* and also from bacteria like *Pseudomonas* and *Azotobacter* as capsular polysaccharide. In 1881, English chemist ECC Stanford with an alkaline solution extracted a

viscous liquid from brown seaweed of *Laminaria* species and called this product as 'Algin'. For the first time studied the properties of alginate. It is also called seaweed gum and plays important role as thickening, stabilizing, gel-forming, and film-forming agent in textile, food, pharmaceutical, product packaging, printing, dyeing and paper industries. Being a natural polymer, alginic acids constitute a family of linear binary copolymers of 1-4 glycosidically linked α-L-guluronic acid (G) and its C-5 epimer β-D-mannuronic acid. (M). The *M G* units may be randomly or non-randomly organized as heterogeneous or homogeneous sequences. Alginates are the salts of alginic acid.100-3,000 units of building block liked together in a stiff and partly flexible chain to form alginate. According to the origin of the alginate: three types of blocks are there homopolymeric M-blocks (M-M), homopolymeric G-blocks (G-G) and heteropolymeric sequentially alternating M-G blocks (M-G). The functional properties of alginate molecules within an encapsulation matrix are related to its composition and block structure of alginate. These are available in sodium, ammonium and potassium derivatives and are soluble in both hot and cold water. The alginate polymer is anionic whenever there are two neighboring guluronic acid residues polyvalent cations bind to the polymer. Thus polyvalent cations are responsible for the cross-linking of both different polymer molecules and within the same polymer chain and helps in giving rise to a three-dimensional network.. The process of gelation, simply takes place by the exchange of calcium ions for sodium ions. The droplet of sodium alginate and cells when dripped into calcium chloride solution form sphere entrapping the cells in a three-dimensional lattice of ionically cross-linked alginate .

$$2\ Na\ (Alginate) + Ca^{++} \text{-------}> Ca\ (Alginate)2 + 2\ Na^{+}$$

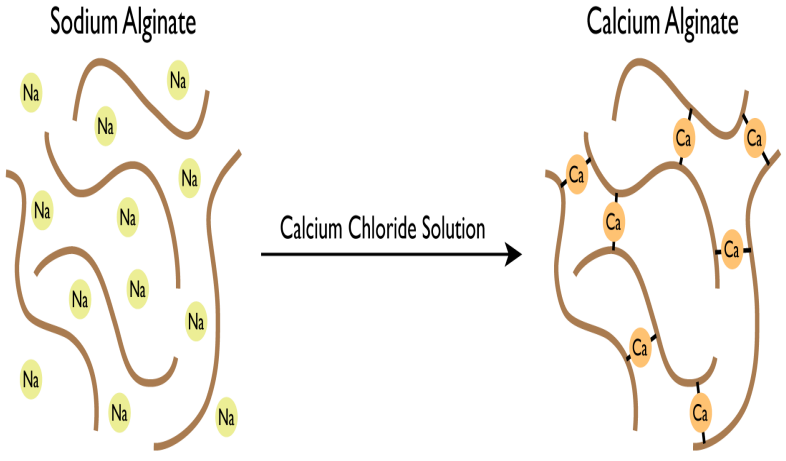

Fig. 2: Polymerization in alginate using calcium chloride

1.5 Immobilization techniques in fermentation industry

Fermentation is a process of incomplete oxidation of substrate i.e. sugar such as glucose, fructose and sucrose in the absence of oxygen. Alcoholic fermentation also referred to as ethanol fermentation is caused by the metabolic activity of yeast cell, which is an anaerobic process. In this process, substrate is converted into cellular energy and produce ethanol and carbon dioxide as metabolic waste product. Now a days fermentation process is used extensively in the biotechnology, pharmaceutical, biofuel, food and beverage industries. Ethanol fermentation increases loaf volume and causes the dough to rise. Alcoholic beverage, fermented food also produced by fermentation by yeast. Bioethanol is an alcohol which is made by yeast through fermentation of carbohydrates such as cellulosic biomass, starch crop etc. Alcohol can be produced by utilizing free cells of yeast or by immobilized yeast cell with in calcium alginate bead. There are various method of ethanol production such as continuous fermentation with cell recycling, vacuum distillation with cell recycling, and immobilization of yeast cell. Alcohol can be produced by utilizing free cells of yeast or by immobilized yeast cell with in calcium alginate bead. Bioreactor productivity, improved cell stability, better substrate utilization are the main objective of immobilization. Fermentation by immobilized cell has many technical and economical advantages as compare to free cell system or traditional method, for example high fermentation rate, simple manner of preparation and handling, better substrate utilization, longer working life time, ease of separation to facilitate their reuse and easy harvest from the product, increased bioreactor productivity, reduces the cost of bioprocessing by eliminating long and expensive processes of cell recovery and cell recycle, maintenance of high cell density per volume, permeable to reactant and product, less inhibition by product, not reduce the desired biocatalytic activity of cell, protect against high shear damage, provide favorable microenvironment to cell, reduce the possibility of contamination, has high tolerance to alcohol etc.. Bead formed by this process are fully active, flexible and hard to withstand mild agitation.

2. Review of literature

2.1 Fermentaion

Two strains of *D. bruxellensis* were capable of producing ethanol at a very high yield that is 0.44 gram per gram of glucose as compare to most well known yeast *Saccharomyces cerevisiae* under oxygen limitation and low pH conditions. The pH at the start of fermentation was 4.5, which gradually decreased to 2.5 at the end of the fermentation (Galafassi et al., 2011). *Saccharomyces cerevisiae* is not able to utilize five carbon sugars that is pentose (Galafassi et al., 2011; Agbogbo et al., 2006). *Candida shehatae, Pachysolen tannophilus, P. stipitis,* are some yeasts, which ferments pentose sugar (Galafassi et al., 2011). Due to the advancement of molecular biology recombinant strains of xylose fermenting *S.cerevisiae* have been proposed (Galafassi et al., 2011). *Dekkera* species have a great potential for the production of ethanol industrially from the renewable source (Galafassi et al., 2011). Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are the two most important fermentative enzymes both in aerobic as well as oxygen limitation condition (Galafassi et al., 2011). The optimum pH range for production of ethanol is pH 4 to pH 5 as in this pH range the amount of by products are less and in between temperature 30°C to 45°C the maximum specific growth rate and ethanol production are observed (Lin et al., 2012). Sugar solution should be concentrated prior to fermentation (Lin et al., 2012). A much high temperature inhibits the growth of cell and decline the fermentation, this may be due to the change in saturation level of soluble compounds, solvents and transport activity in the cell which lead to accumulation of toxin within the cell and also due to the denaturation of ribosomes and enzymes (Lin et al., 2012). The ideal temperature for fermentation is believed to be in the range 20°C to 35°C and increase in temperature beyond it causes problem in fermentation (Lin et al., 2012). Yeast becomes inactive due to low tolerance to ethanol at low temperature (Lin et al., 2012). A high substrate concentration prevents the ethanol fermentation as pH is changed due to the accumulation of high concentration of ethanol and byproducts (Lin et al., 2012). *Candida shehatae, Pachysolen tannophilus, P. stipitis* are the yeast which are capable of fermenting both glucose and xylose to ethanol (Agbogbo et al., 2006; Lynd et al., 1999; Bothast and Saha, 1997; Schneider et al., 1981). *P. stipitis* is capable of fermenting a wide range of sugars with high ethanol yield without requirement of vitamins. (Agbogbo et al., 2006;

Preez and Prior, 1985). *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the most important microorganism for producing bioethanol (Flickinger and Drew, 1999).

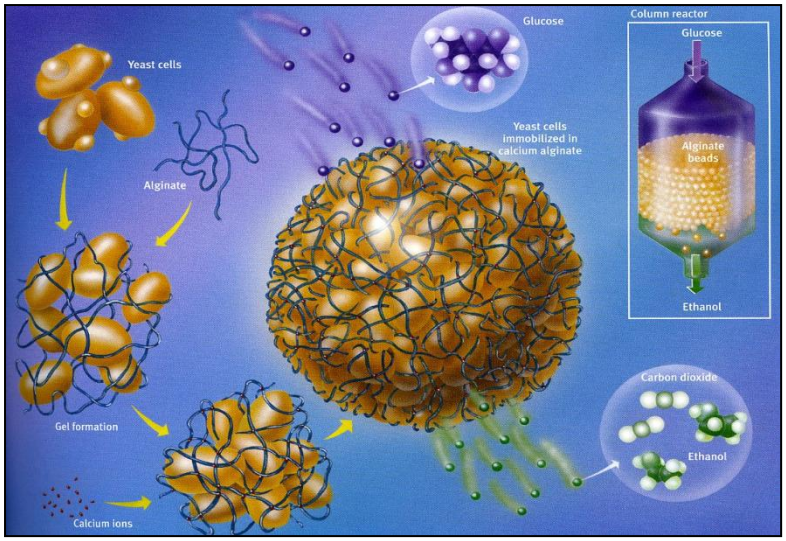

Fig. 3: Use of immobilized yeast cells in ethanol fermentation

2.2 Immobilization

Immobilization increases the productivity of bioreactor, improves cell stability, provides better substrate utilization and decreases the start up time (Ghorbani et al., 2011). Entrapment with in porous matrix, adsorbtion, and encapsulation are the common methods of immobilization (Calinescu et al., 2012). Alginate is most commonly used in immobilization as it is simple, nontoxic, and cheap. So it is mostly used in fermentation industries, pilot plant and in laboratories (Ciesarova et al., 1998). Calcium alginate is the natural polymer mostly used for immobilization of cells (Calinescu et al., 2012). It is the best matrix as it does not change the structure and activity of enzymes within the bead. The stability of alginate is increased by addition of gelatin in calcium alginate bead but the efficiency of fermentation is decreased (Calinescu et al., 2012). The bead size cannot be less than 200 micrometer in the droplet method (Liu et al., 2001). Encapsulation protect the bacteria from adverse environmental conditions (Anal and Singh, 2007; Champagne and Fustier, 2007). Dripping by gravity is the simplest method to make bead and the size of the bead will depend upon it's weight, surface tension and nozzle perimeter and higher than 2 mm bead can be made by this process . When the calcium ion binds between the G block of alginate it forms a three dimensional network and then gelling

occurs (Chávarri et al., 2012). Bacterial culture (10^7 cfu/ml), Starch (10g/l), alginate (18g/l), 0.1 mol/l calcium chloride solution is used for encapsulation. The immobilized cells are released from the alginate bead by dissolving them in 0.1 m/l phosphate buffer and the enumeration was done after 0, 3, 5, 7 week of storage (Kailasapathy, 2005). Microencapsulation increases the survivability of probiotic cultures as compared to free cells in yogurts, which is stored for over 7 weeks. It does not change the sensory properties like colour, flavor, taste but changed the smoothness of yoghurt (Kailasapathy, 2005). Immobilized yeast made by calcium alginate produces more ethanol by consuming more sugar as compared to free yeast cell under the same condition (30°C during fermentation, pH 5, 10 % glucose concentration and 2% sodium alginate concentration). The fermentation time in case of free cell was 36 hour that was more than the fresh (24 hour) and reused bead (10-14 hour). Immobilized yeast produced 100% ethanol as compare to free cell that produced 88% ethanol (Lee et al., 2011). Immobilized yeast can be easily separated from ethanol so that they can be reused for further cycle of fermentation. Immobilization offers numerous advantages over the traditional method of fermentation. Small reactor size is required during immobilization. Immobilized cell can tolerate high concentration of ethanol, fermentation time can be shortened and its productivity increased, less cost of recover, cell stability and activity is also increased (Kourkoutas et al., 2004; Tata et al., 1999). *S.cerevisiae* have various application in food and drink industry due to its invertase enzyme (Buchholz, Kasche, & Bornscheuer, 2005). In hollow-fiber membrane bioreactors baker's yeast Saccharomyces cerevisiae was immobilized(Inloes et al.,1983).The Ultrastructural characteristics of calcium alginate bead and the alginate matrix is called as egg box model by TEM (Wright,2000). The inoculums media for growing of *Saccharomyces cerevisiae* contains 1 gram each of peptone and yeast extract, 5 gram glucose, 0.1 gram each of $MgSO_4$ and KH_2PO_4 in100 ml of deionised water. Fermentation media contains 10% glucose, 5 gram each of peptone and yeast extract, 1 gram each of MgSO4 and K_2PO_4 in 1 liter distilled water. Then the bead were prepared with 2% sodium alginate solution mixed with 24 hour grown culture and by dripping them in 0.1m calcium chloride solution. After that the bead were washed with deionised water to remove the excess calcium chloride. Then before fermentation beads were stored in deionised water for 3 days at 4°C, after that bead were added to fermentation media. Glucose and ethanol concentration was measured by DNSA and Gas chromatography respectively. As the sodium alginate concentration increases there is delay in consumption of glucose because at higher

concentration glucose cannot easily enter in to cell pore (Lee et al., 2011). Beads of larger diameter are more prone to as compared to beads of smaller size (Gilson and Thomas, 1995).The colour and diameter of the bead are different before and after fermentation. Fresh beads are of white colour where as reused beads are of yellow colour, this is due to the difference in the yeast concentration between the two bead samples. Beads also swell after fermentation for example from 3.8mm to 4.4mm diameter. Yeast cells are scattered across the surface in the fresh bead and some yeast are also agglutinated at the middle but in the reused beads yeast cells are densely covered. According to the TEM images the yeast cells are more in reused beads than in the fresh beads. With the increase of bead diameter the ethanol yield and consumption of glucose get decreased (Lee et al., 2011).Yeast cell immobilization using porous alginate have a greater application in the field of biomedical and pharmaceutical industries (Eiselt et al., 2000; Li et al., 1994). Alginate beads errupt and cells are released from the core by the massive release of carbon dioxide (Rakin et al., 2009; Tataridis et al., 2005; Godia et al., 1991). In relation to productivity and stability of enzyme activities immobization is more advantageous than the free cell system (Dorota et al., 2013).

3. Objectives

3.1 To immobilize yeast cells using the calcium alginate bead.

3.2 To utilize these immobilized yeast cells in alcohol fermentation.

3.3 To study the effect of immobilization on ethanol production.

3.4 To study the effect of storage at different temperature on viability of yeast cell.

4. Materials and Methods

4.1 Culture maintenance

Three yeast strains i.e. *Saccharomyces cerevisiae* NCIM 3570, *Candida shehatae* NCIM 3500 and *Dekkera naardenensis* NCIM 3575 was purchased from NCIM (National Collection of Industrial Microorganisms), National Chemical Laboratory, Pune. These cultures were maintained on MGYP (Malt extract 1%, glucose 1%, yeast extract 0.3%, peptone 1%) medium at pH 6.0.

4.2 Immobilization of yeast cells using calcium alginate beads

Yeast cells were cultured on MGYP broth at pH 6.0, for 48 h at 30°C at 120 rpm. Three percent sodium alginate and 0.05 M calcium chloride solution was prepared using deionised water and both the solutions were autoclaved at 121^{o}C (15 psi) for fifteen minutes. To the autoclaved sodium alginate solution, 48 h old yeast culture was added in 1:1 (V/V) and the solution was homogenized using vortex. This solution was added drop wise using a syringe with needle diameter of 1 mm to the calcium chloride solution. The capsules/beads formed were allowed to harden for 10 minutes, washed in deionized water twice and store in aseptic falcon tubes for future studies (Manasouripour et al., 2013).

4.3 Utilization of the immobilized yeast cells in ethanol production

Immobilized yeast cells obtained in the form of beads were utilized for fermentation of sugar solution. Fermentation media was prepared using 10% initial glucose concentration for *S.cerevisiae* and *D. naardenensis* at pH 6.0-6.5 and 5%glucose to 5% xylose concentration for *C. shehatae* at pH 5.5. 10 % of inoculum (immobilized and free cells) was added to fermentation media. Fermentation media was incubated for 120 h at 30°C at 120 rpm. After incubation, the samples were distilled using rotary evaporator for separation of ethanol from media.

4.4 Estimation of ethanol production by HPLC analysis

Amount of ethanol in the distillate was estimated by HPLC analysis using Hiplex-H Agilent column having Column and Detector Temperature 57°C and 50°Crespectively, flow rate 0.7 ml/min, mobile phase 1 mM H_2SO_4 using refractive index detector.

4.5 Storage of immobilized yeast cells under variable conditions

Immobilized yeast cell were stored in different condition like at 4°C, at room temperature and in dried form using aseptic falcon tubes properly sealed with parafilm strips.

Immobilized yeast cells used in fermentation were also harvested, washed with autoclaved distilled water and stored in aseptic condition at 4°C to check the viability and culture load.

4.6 Viability of immobilized yeast cell stored under different storage conditions

The entrapped bacterial cells were released from the capsules using 0.2 M/l phosphate buffer. To count the encapsulated bacteria 1g sample was re-suspended in 9ml of 0.2 mol/l phosphate buffer in a aseptic falcon tube. It was vortexed for 20 min to allow complete release of the yeast cells from alginate capsules. Sample so obtained was serially diluted in autoclaved distilled water and plated on MGYP agar plates. All samples were analysed in triplicates (Godward & Kailasapathy, 2005).

4.7 Field Emission Scanning Electron Microscopy images of yeast cells

Three yeast strains i.e. *Saccharomyces cerevisiae NCIM 3570*, *Candida shehatae NCIM 3500* and *Dekkera naardenensis NCIM 3575* were subjected to SEM imaging to study the morphology. All the three strains were cultured for 24 h at 30°C at120 rpm. Culture samples were centrifuged at 5000 rpm for 15 min. and washed twice with PBS. The final pellets were suspended in PBS and vortexed. Slides were fixed using 2.5% glutaraldehyde for 16 h and then with 1% tannic acid for 5 min. Slides were then washed with distilled water and dehydrated by using 30%, 50%, 70%, 90% and absolute ethanol in a series.

5. Results and Discussion

5.1 Immobilization of yeast cells using calcium alginate beads

By using 3% sodium alginate and 0.5M $Cacl_2$ solution stable, active beads are produced in case of all the three yeast strain that are *S. cerevisiae, C. shehatae and D. naardenensis*. The beads are of same size, same diameter and are round in nature (Fig. 4).

Fig 4: Immobilized yeast cells

5.2 Utilization of the immobilized yeast cells in ethanol production

Immobilized cells in ethanol production media retain their shape and not degraded during the fermentation process. Fermentation media in case of *C. shehatae* was showing dark colour than *S. cerevisiae* and *D. naardenensis* due to the presence of both glucose and xylose in the media component. Presence of bubble formation in the sealed conical flask during the fermentation process confirms the production of CO_2 and thus ethanol (Fig. 5).

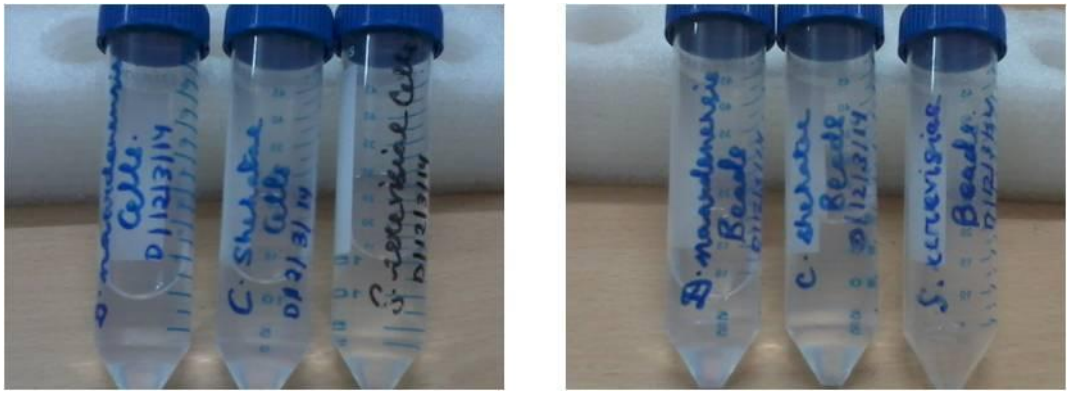

Fig 5: Distilled ethanol produced from both free and encapsulated yeast cell

5.3 Estimation of ethanol production by HPLC analysis

Ethanol production was estimated in High Performance Liquid Chromatography in National Institute of Renewable Energy, Kapurthala, Punjab. The HPLC Column used here was HIPlex-H Agilent having column temperature and detector temperature 57°C and 50°C respectively, by using refractive index detector where the flow rate was 0.7 ml/min and mobile phase was 1 mM H_2SO_4. The produced ethanol shows peak at retention time of 18.850 min (Fig. 6).

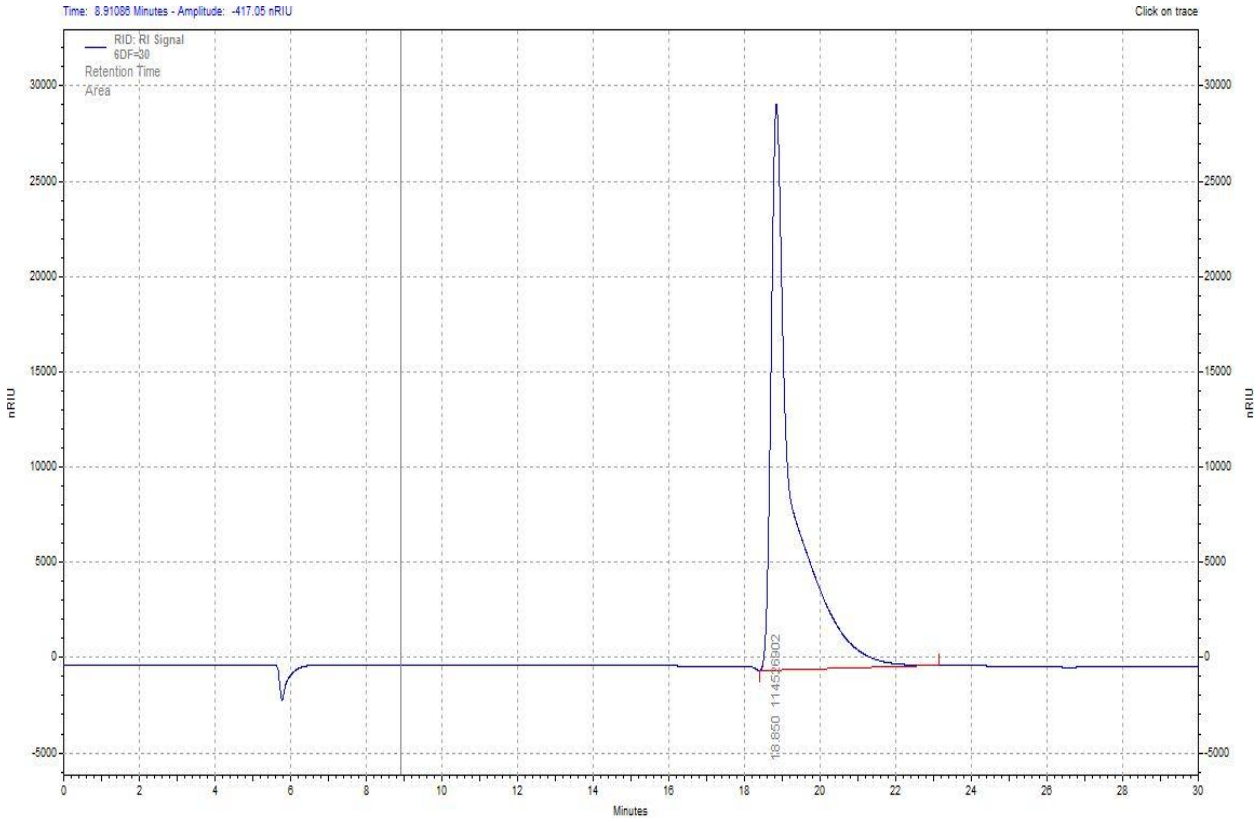

Fig 6: HPLC Chromatogram for standard ethanol.

The amount of ethanol produced in immobilized *S.cerevisiae* is 194.69 g/l and incase of free cell it is 81.24 g/l (Table 1). In case of encapsulated condition, the cells have a favorable microenvironment and more active than free cell. However, in case of *C. shehatae and D. naardenensis* the free cell are showing good result than the immobilized condition. Here also, the entrapped cells are in favorable condition and active but the problem was lies in distillation. All a result what we are getting here are only after distillation of fermented broth in rotary evaporator, due to that some amount of ethanol is lost during the distillation process. So that it gave less

concentration of ethanol in case of *C. shehatae and D. naardenensis*. But in case of *S.cerevisiae* it did not cause that much reduction in ethanol concentration since it produces more ethanol during the fermentation process.

Table 1: Amount of ethanol estimated using Hiplex-H Agilent column

S.No.	Samples	Ethanol Concentration (g/L)
1	Immobilized S. cerevisiae	194.69
2	Free S.cerevisiae	81.24
3	Immobilized C. shehatae	45.08
4	Free C. shehatae	60.69
5	Immobilized D. naardenensis	102.70
6	Free D. naardenensis	306.02

5.4 Storage of immobilized yeast cells under variable conditions

Beads do not show any contamination or degradation after 45 days of storage in either 4°C or room temperature condition. Used beads, which were stored at 4°C, are swollen after their use in first cycle of fermentation due to enlargement of pore size. It signifies that nutritive media component as well as substrate has gone into the bead and products like ethanol, CO_2 have come out from the bead. The colors of the used beads are different from the colour of their respective fresh beads (Fig. 7).

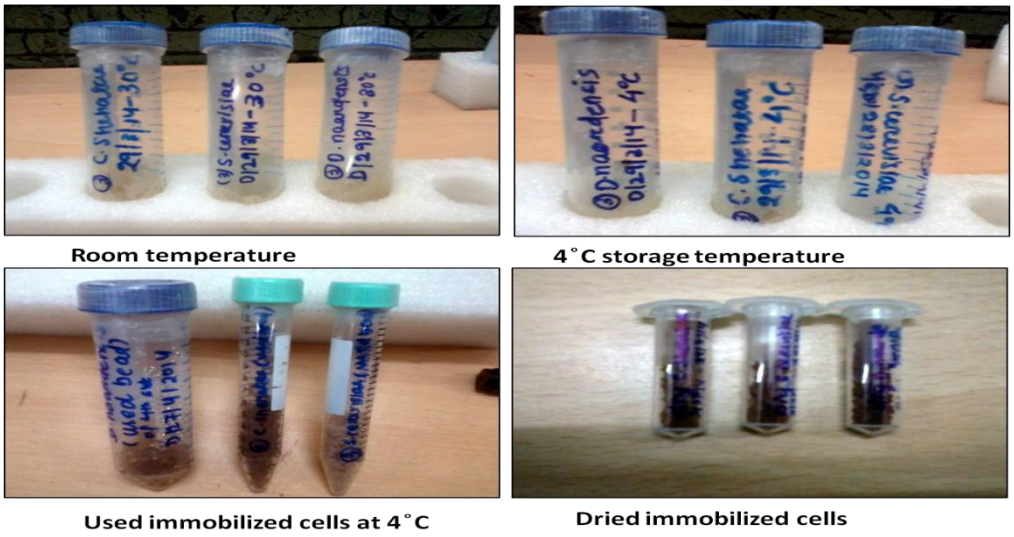

Fig. 7: Stored alginate beads with immobilized yeast cells under room temperature, 4°C and in dried condition

5.5 Viability of immobilized yeast cell stored under different storage conditions

After 45 days of storage period, viable yeast colonies were evidenced on MGYP agar plates. Used beads were also showing good but lesser number of colonies than 4°C and room temperature stored beads. It suggests that the cells within the beads are viable and they can be used and reused in the fermentation process (Table 2).

5.6 Field Emission Scanning Electron Microscopy images of three yeast cells

Morphological features of the yeast cells were observed using FE SEM. *C. shehatae* and *D. naardenensis* single cell images were taken in 50,000X magnification where as in case of *S. cerevisiae* it was 20,000X (Fig. 9).

Table 2: Cell viability of immobilized yeast cells under different storage conditions

S.No.	Yeast Strains	Storage Conditions	CFU /mg
1	*S. cerevisiae*	4 □C	298
2	*S. cerevisiae*	30 □C	178
3	*S. cerevisiae*	Used	167
4	*C. shehatae*	4 □C	238
5	*C. shehatae*	30 □C	135
6	*C. shehatae*	Used	85
7	*D. naardenensis*	4 □C	227
8	*D. naardenensis*	30 □C	213
9	*D. naardenensis*	Used	106

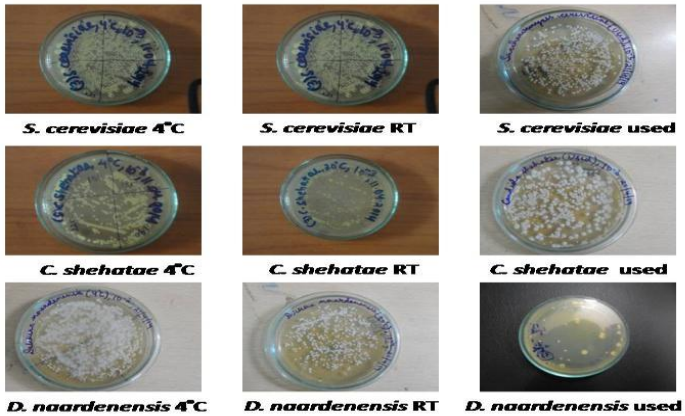

Fig. 8: MGYP agar plates showing viable yeast colonies under 4°C, room temperature and in use form stored at 4°C

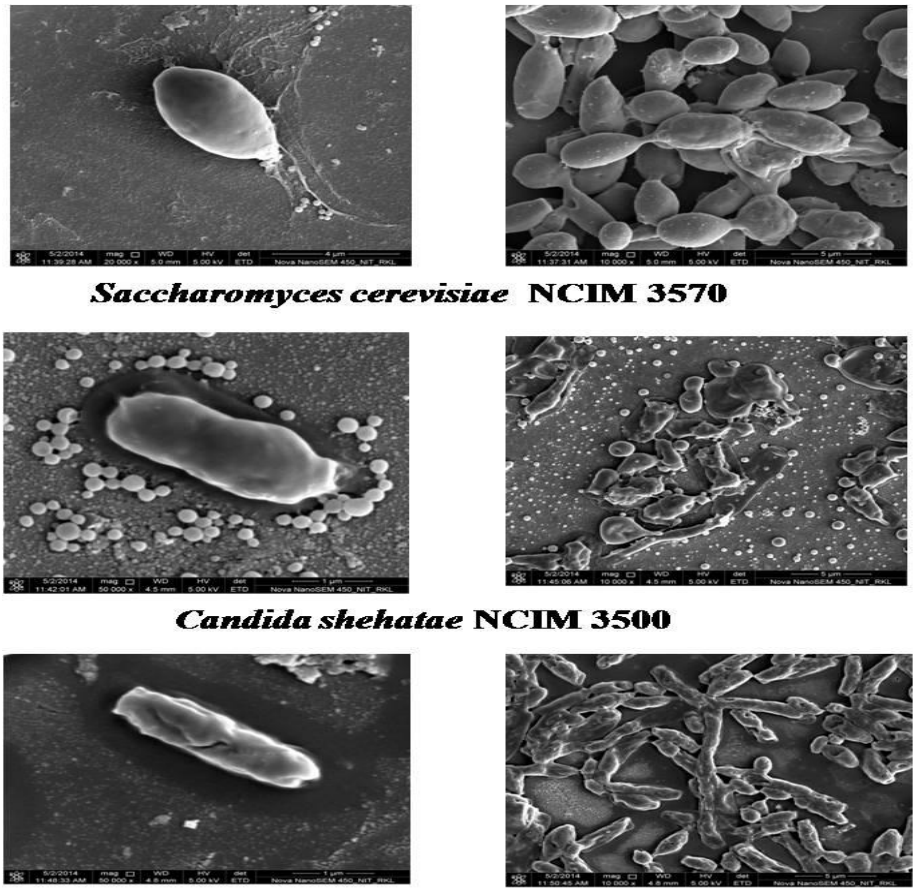

Fig. 9: Field Emission Scanning Electron Microscopy (FESEM) images of three yeast cells

6. Conclusion

The encapsulation of three yeast cells *Saccharomyces cerevisiae, Candida shehatae and Dekkera naardenensis* in calcium alginate beads is the most suitable and efficient process of immobilization of yeast cells. This immobilized yeast cells have grater application industrially in the production of ethanol and many more by products. If these immobilized yeast cells are used in industries like baking, brewing, food etc., the production rate will surely increase as well as it will be cost effective and favor reuse. Immobilized yeast cells produce higher ethanol as compare to free cells and having various benefits over it like it have rapid fermentation time, fewer by products and no residual glucose content that is present on initial fermentation broth. For the economical ethanol production utilization of these yeast cells in the fermentation broth is the best method. Raw biological substrates like lignocelluloses, agricultural wastes can be utilized in place of pure substrates like glucose, xylose. Thus Bioethanol produced from this have greater role in bio fuel industries which will meet the demand of energy of human being In the present study, they are showing good storage capabilities, so they can be stored for many days and reused repeatedly.

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